

An intercomparison of HPLC phytoplankton pigment methods using in situ samples: application to remote sensing and database activities

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Abstract

Whether for biogeochemical studies or ocean color validation activities, high-performance liquid chromatography (HPLC) is an established reference technique for the analysis of chlorophyll *a* and associated phytoplankton pigments. The results of an intercomparison exercise of HPLC pigment determination, performed for the first time on natural samples and involving four laboratories (each using a different HPLC procedure), are used to address three main objectives: (a) estimate (and explain) the level of agreement or discrepancy in the methods used, (b) establish whether or not the accuracy requirements for ocean color validation activities can be met, and (c) establish how higher order associations in individual pigments (i.e., sums and ratios) influence the uncertainty budget while also determining how this information can be used to minimize the variance within larger pigment databases. The round-robin test samples (11 different samples received in duplicate by each laboratory) covered a range of total chlorophyll *a* concentration, [TChl *a*], representative of open ocean conditions from 0.045 mg m⁻³, typical of the highly oligotrophic surface waters of the Ionian Sea, to 2.2 mg m⁻³, characteristic of the upwelling regime off Morocco. Despite the diversity in trophic conditions and HPLC methods, the agreement between laboratories, defined here as the absolute percent difference (APD), was approximately 7.0% for [TChl *a*], which is well within the 25% accuracy objective for remote sensing validation purposes. For other pigments (mainly chemotaxonomic carotenoids), the agreement between methods was 21.5% on average (ranging from 11.5% for fucoxanthin to 32.5% for peridinin), and inversely depended on pigment concentration (with large disagreements for pigments close to the detection limits). It is shown that better agreement between methods can be achieved if some simple procedures are employed: (a) disregarding results less than the effective limit of quantitation (LOQ, an alternative to the method detection limit, MDL), (b) standardizing the manner in which the concentration of pigment standards are determined, and (c) accurately accounting for divinyl chlorophyll *a* when computing [TChl *a*] for those methods which do not chromatographically separate it from monovinyl chlorophyll *a*. The use of these quality-assurance procedures improved the agreement between methods, with average APD values dropping from 7.0% to 5.5% for [TChl *a*] and from 21.5% to 13.9% for the principal carotenoids. Additionally, it is shown that subsequent grouping of individual pigment concentrations into sums and ratios significantly reduced the variance and, thus, improved the agreement between laboratories. This grouping, therefore, provides a simple mechanism for decreasing the variance within databases composed of merged data from different origins. Among the recommendations for improving database consistency in the future, it is suggested that submissions to a database should include the relevant information related to the limit of detection for the HPLC method.

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1. Introduction

Because phytoplankton concentration is an important variable in the study of marine biogeochemical cycles, the accurate quantification of its biomass is a fundamental requirement. Phytoplankton biomass is typically approximated by quantifying chlorophyll *a* concentration, [Chl *a*], for which many methods ranging from the single cell to the synoptic (remote sensing) scale have been developed (Yentsch and Menzel, 1963; Parsons and Strickland, 1963; Olson et al., 1983; O'Reilly et al., 1998).

The taxonomic composition of phytoplankton influences many biogeochemical processes, so it is essential to simultaneously determine phytoplankton biomass and composition over the continuum of phytoplankton size (approximately 0.5–100 μm). The determination of chlorophyll and carotenoid pigment concentrations by high-performance liquid chromatography (HPLC) is a method which fulfills most of these requirements. Indeed, many carotenoids and chlorophylls are taxonomic markers of phytoplankton taxa, which means community composition can be evaluated at the same time that [Chl *a*] is accurately quantified.

Since the initial methodological paper by Mantoura and Llewellyn (1983), the possibility of determining community composition and biomass has resulted in the HPLC method rapidly becoming the technique of choice in biogeochemical and primary production studies. The use of HPLC methods in marine studies has also been promoted, because the international Joint Global Ocean Flux Study (JGOFS) program recommended HPLC in the determination of [Chl *a*] (JGOFS, 1994) and, more precisely, to use the protocol of Wright et al. (1991). Since the start of the JGOFS decade in the 1980s, HPLC techniques have evolved considerably (Jeffrey et al., 1999), and some JGOFS contributors decided not to follow the original JGOFS recommendation in order to take full benefit of the ongoing methodological evolutions. In particular, the C8 method of Goericke and Repeta (1993) was an important improvement, because it allowed the separation of divinyl chlorophyll *a* from its monovinyl form. Subsequent adaptations of this method were proposed (e.g., Vidussi et al., 1996; Barlow et al., 1997) and used for a variety of JGOFS cruises. More recently, new methods have also been proposed that rely on C8 phase and elevated column temperature to achieve the desired separation selectivity (Van Heulekem and Thomas, 2001) or on mobile phase modified with pyridine to resolve chlorophyll *c* pigments (Zapata et al., 2000).

5. Conclusions

This study was designed to intercompare, within a round-robin context, the HPLC determination of pigments useful to biogeochemical studies, as well as the uncertainty requirements for validating the SeaWiFS remote sensing chlorophyll *a* product. This activity

was expected to estimate the uncertainties in the HPLC method used and give reasons for the level of agreement (or discrepancy) achieved. The analysis was also designed to quantify how methodological uncertainties propagate within a database originating from different sources and demonstrate how this information can be exploited.

Some HPLC procedures reduced the variance for individual pigments. These procedures included disregarding results less than the effective LOQ (a method detection limit alternative), standardizing the manner in which the concentration of pigment standards are determined, and accurately accounting for [DVChl *a*] when computing [TChl *a*] in cases where [DVChl *a*] is present in significant proportions. When data reported by laboratories were modified according to these procedures, the average uncertainty across all laboratories (for all individual pigments) was reduced by about 6.6% (from 22.2% to 15.6%). Notably, the uncertainty in determining [TChl *a*] (0.045–2.200 mg m^{-3}) was only 7.0%, which was reduced to 5.5% when all laboratories individually quantified [DVChl *a*]. These findings suggest that, on average, it is possible for HPLC techniques to meet uncertainty objectives for ocean color validation activities and that HPLC quality-assurance practices are important to the reduction of uncertainty levels.

Another mechanism for reducing the uncertainties is to form the individual pigments into higher order variables. In every case, the average APD decreased as pigments were grouped from individual concentrations into sums and then ratios, with the latter showing the most robust statistical properties. The macrovariables always performed as well as the sums. This strongly suggests that as larger databases are constructed from incremental contributions (i.e., from different geographic locations and analysts), the most reliable use of the data will likely be with the higher order variables. Furthermore, the low uncertainties (and standard deviations) with the pigment ratios indicate they can also be used for quality-assurance purposes (at least in open ocean data).

Finally, the reproduction of the principal aspects of the baseline analysis with the alternative approaches (using the round-robin data set as a model for the entire PROSOPE data set) suggests the round-robin data represent an acceptable subsampling of the conditions encountered during the PROSOPE cruise. This confirms the results achieved with a statistically valid subset can be applied successfully to a superset and justifies a recommendation for a group of laboratories to incrementally analyze a set of replicates from a variety of field campaigns over time. A continuing series of round-robins based on diverse geographical samples would provide a better statistical description of how uncertainties propagate through increasingly extensive databases, particularly as a function of trophic levels and methodologies.